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June 26, 2007

CONTAIN NO CBI

TSCA Document Processing Center (7407W)
U.S. Environmental Protection Agency
ATTN: TSCA Section 8(e) Coordinator
Office of Pollution Prevention and Toxics
1200 Pennsylvania Avenue, NW
Washington, DC 20460-0001

Re: 8EHQ-07-16748 – TSCA 8(e) Substantial Risk Notification 1,5-Cyclooctadiene (CAS #111-78-4) - Final Report

Dear 8(e) Coordinator:

INVISTA is submitting the final study report on 1,5-Cyclooctadiene (COD), CASRN 111-78-4, conducted by SafePharm Laboratories in the United Kingdom. INVISTA had indicated in its February 28, 2007 TSCA 8(e) notification that upon the completion and acceptance, we would forward a copy of the final report for the ACUTE TOXICITY TO *DAPHNIA MAGNA* study for 1,5-Cyclooctadiene (COD) to your office.

The final study has the same findings as the previous draft results submitted by INVISTA. These results are:

The acute toxicity (48 hr EC_{50}) of COD to the crustacean, *Daphnia magna*, was reported to be 0.13 mg/l [95% Confidence Limits of 0.10 – 0.16 mg/l] in an OECD 202 assay.

If you have any questions or need additional information, please contact me at (316) 828-1470.

Sincerely,

Betsy Duncan

8EHQ-07-16748

Betsy Duncan
TSCA Program Manager
Environmental Health and Safety

Attachment

8907000038

305681

SafePharm Laboratories

1,5-Cyclooctadiene (COD):

ACUTE TOXICITY TO *DAPHNIA MAGNA*

SPL PROJECT NUMBER: 2231/0009

AUTHORS: T J Goodband
D Mullee

SPONSOR:

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QUALITY ASSURANCE REPORT

This study type is classed as short-term. The standard test method for this study type ("General Study Plan" in OECD terminology) was reviewed for compliance once only on initial production. Inspection of the routine and repetitive procedures that constitute the study is carried out as a continuous process designed to encompass the major phases at or about the time this study was in progress.

This report has been audited by Safepharm Quality Assurance Unit, and is considered to be an accurate account of the data generated and of the procedures followed.

In each case, the outcome of QA evaluation is reported to the Study Director and Management on the day of evaluation. Audits of study documentation, and process inspections appropriate to the type and schedule of this study were as follows:

	15 February 2005	Standard Test Method Compliance Audit
	13, 19 December 2006	Test Material Preparation
	13 December 2006	Test System Preparation
	13 December 2006	Exposure
	19 December 2006	Assessment of Response
	05, 07, 18 December 2006	Chemical Analysis
§	21 February 2007	Draft Report Audit
§	Date of QA Signature	Final Report Audit

§ Evaluation specific to this study

..... DATE: 04 JUN 2007

For Safepharm Quality Assurance Unit*

***Authorised QA Signatures:**

Head of Department:	JR Pateman CBiol MIBiol DipRQA AIQA FRQA
Deputy Head of Department:	JM Crowther MIScT MRQA
Senior Audit Staff:	JV Johnson BSc MRQA; G Wren ONC MRQA

GLP COMPLIANCE STATEMENT

The work described was performed in compliance with UK GLP standards (Schedule 1, Good Laboratory Practice Regulations 1999 (SI 1999/3106 as amended by SI 2004/0994)). These Regulations are in accordance with GLP standards published as OECD Principles on Good Laboratory Practice (revised 1997, ENV/MC/CHEM(98)17); and are in accordance with, and implement, the requirements of Directives 2004/9/EC and 2004/10/EC.

These international standards are acceptable to the Regulatory agencies of the following countries: Australia, Austria, Belgium, Canada, the Czech Republic, Denmark, Finland, France, Germany, Greece, Hungary, Iceland, Ireland, Israel, Italy, Japan, Republic of Korea, Luxembourg, Mexico, The Netherlands, New Zealand, Norway, Poland, Portugal, Slovenia, South Africa, Spain, Sweden, Switzerland, Turkey, the United Kingdom, and the United States of America.

This report fully and accurately reflects the procedures used and data generated.

..... Date: 04 JUN 2007

T J Goodband BSc
Study Director

The analytical data presented in this report were compiled by me or under my supervision and accurately reflect the data obtained.

..... Date: 04 JUN 2007

D Mullee CChem MRSC
Director of Analytical Services

CONTENTS

1,5-Cyclooctadiene (COD):

ACUTE TOXICITY TO *DAPHNIA MAGNA*

SUMMARY

Introduction. A study was performed to assess the acute toxicity of the test material to *Daphnia magna*. The method followed that described in the OECD Guidelines for Testing of Chemicals (April 2004) No 202, "*Daphnia* sp, Acute Immobilisation Test" referenced as Method C.2 of Commission Directive 92/69/EEC (which constitutes Annex V of Council Directive 67/548/EEC).

Methods. Information supplied by the Sponsor indicated that the test material forms peroxides when in contact with air. Initial stability analysis suggested that the test material was stable in the light however, information pertaining to the test material suggested that light conditions may accelerate peroxide formation. Therefore the test material was weighed out and prepared under non-actinic light using completely filled glass stoppered conical flasks in order to minimise any losses.

Following a preliminary range-finding test, twenty daphnids (2 replicates of 10 animals) were exposed to an aqueous solution of the test material at time-weighted mean measured concentrations of 0.0053, 0.0080, 0.010, 0.033, 0.036, 0.065, 0.15, 0.29 and 0.58 mg/l for 48 hours at a temperature of approximately 20°C under static test conditions. The test material solutions were prepared by shaking an excess (300 mg/500 ml) of test material in reconstituted water at approximately 200 rpm at a temperature of 30°C for 24 hours. After the 24-Hour shaking period the mixture was allowed to cool to approximately 20°C and any undissolved test material was removed by filtration (0.2 µm Gelman AcroCap filter) to produce the saturated solution (103 mg/l based on the chemical analysis at the start of the test). This was then further diluted, as necessary, to provide the remaining test groups. The number of immobilised *Daphnia* were recorded after 24 and 48 hours.

A positive control conducted approximately every six months used potassium dichromate as the reference material. *Daphnia magna* was exposed to an aqueous solution of the reference material at concentrations of 0.32, 0.56, 1.0, 1.8 and 3.2 mg/l for 48 hours at a temperature of 20.0°C to 21.1°C under static test conditions. Immobilisation and any adverse reactions to exposure were recorded after 3, 24 and 48 hours.

Results Analysis of the saturated solution at 0 hours showed a measured concentration of 103 mg/l.

Analysis of the test preparations at 0 hours showed measured test concentrations to range from 0.0063 to 0.66 mg/l and after 48 hours to range from 0.0044 to 0.51 mg/l. This decline in prepared test concentrations at 0 hours was considered to be due to the volatility of the test material although every precaution was taken to minimise any losses.

Given this decline in measured test concentrations over the 48-Hour period it was considered justifiable to base the results on the time-weighted mean measured test concentrations of the test media in order to give a "worst case" analysis of the data. The 48-Hour EC_{50} based on the time weighted mean measured test concentrations of the test media was 0.13 mg/l with 95% confidence limits of 0.10 - 0.16 mg/l and the No Observed Effect Concentration was 0.036 mg/l.

The 48-Hour EC_{50} for the reference material to *Daphnia magna* based on nominal concentrations was 0.85 mg/l with 95% confidence limits of 0.76 - 0.96 mg/l. The No Observed Effect Concentration was 0.56 mg/l.

1,5-Cyclooctadiene (COD):

ACUTE TOXICITY TO *DAPHNIA MAGNA*

1. INTRODUCTION

This report contains a description of the methods used and results obtained during a study to investigate the acute toxicity of the test material to *Daphnia magna*. The method followed the recommendations of the OECD Guidelines for Testing of Chemicals (April 2004) No 202 "*Daphnia* sp, Acute Immobilisation Test" referenced as Method C.2 of Commission Directive 92/69/EEC (which constitutes Annex V of Council Directive 67/548/EEC).

Daphnia magna is a freshwater invertebrate representative of a wide variety of natural habitats, and can therefore be considered as an important non-target organism in freshwater ecosystems.

The range-finding test was conducted between 8 November 2006 and 11 November 2006 and the definitive test between 11 December 2006 and 14 December 2006.

The positive control (Safepharm Laboratories Project No: 0039/0874) was conducted between 30 October 2006 and 01 November 2006.

In view of the difficulties associated with the evaluation of aquatic toxicity of poorly water soluble test materials, a modification of the standard method for the preparation of aqueous media was performed. An approach endorsed by several important regulatory authorities in the EU and elsewhere (ECETOC 1996, and OECD 2000), is to expose organisms to a saturated solution of the test material in cases where the test material is of high purity and is poorly soluble in water and in the permitted auxiliary solvents and surfactants. Using this approach, a saturated solution was prepared by shaking an excess (300 mg/500ml) of the test material in reconstituted water for a period of 24 hours prior to removal of any undissolved test material by filtration (0.2 μm) to give a saturated solution.

2. TEST MATERIAL AND EXPERIMENTAL PREPARATION

2.1 Description, Identification and Storage Conditions

Sponsor's identification	:	1,5-Cyclooctadiene (COD)
Description	:	colourless liquid
Batch number	:	06010MD
Date received	:	28 June 2006 and 10 August 2006
Storage conditions	:	room temperature in the dark under nitrogen

The integrity of supplied data relating to the identity, purity and stability of the test material is the responsibility of the Sponsor.

2.2 Experimental Preparation

Information supplied by the Sponsor indicated that the test material may be light sensitive therefore all test media preparations were carried out under a non-actinic light source and the test vessels were shielded from the light.

The test concentrations used in the definitive test were prepared from a saturated solution prepared from an initial test material dispersion of 300 mg/500 ml.

An amount of test material (300 mg) was dispersed in 500 ml of reconstituted water and shaken at approximately 200 rpm at a temperature of 30°C for approximately 24 hours. After 24 hours the shaking was stopped and the mixture allowed to cool to approximately 20°C. Any undissolved test material was removed by filtration (0.2µm Gelman AcroCap filter, first approximate 100 ml discarded) to give the 103 mg/l* saturated solution.

Aliquots (0.20, 0.36, 0.64, 1.12, 2.0, 3.6, 6.4, 11.2 and 20 ml) of this 103 mg/l saturated solution were each separately dispersed in a final volume of 2 litres of reconstituted water to give the 0.0053, 0.0080, 0.010, 0.033, 0.036, 0.065, 0.15, 0.29 and 0.58 mg/l** test concentrations respectively.

Each stock prepared concentration was inverted several times to ensure adequate mixing and homogeneity.

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* Based on chemical analysis of the saturated solution prepared for the definitive test.
** Time-weighted mean measured test concentrations at the end of the test.

The concentration and stability of the test material in the test preparations were verified by chemical analysis at 0 and 48 hours (see Appendix 2).

2.3 Reference Material

A positive control (Safepharm Laboratories Project No: 0039/0874) conducted approximately every six months used potassium dichromate (Sigma Lot No 092K0203) as the reference material. An amount of reference material (100 mg) was dissolved in reconstituted water and the volume adjusted to 1 litre to give a 100 mg/l stock solution. An aliquot (50 ml) of this stock solution was diluted in reconstituted water and the volume adjusted to 500 ml to give a 10 mg/l stock solution. Aliquots (16, 28, 50, 90 and 160 ml) of the 10 mg/l stock solution were each separately dispersed in a final volume of 500 ml of reconstituted water to give the test series of 0.32, 0.56, 1.0, 1.8 and 3.2 mg/l.

Each stock solution and prepared concentration was inverted several times to ensure adequate mixing and homogeneity.

3. METHODS

3.1 Test Species

The test was carried out using 1st instar *Daphnia magna* derived from in-house laboratory cultures.

Adult *Daphnia* were maintained in polypropylene vessels containing approximately 2 litres of reconstituted water in a temperature controlled room at 18.2 to 18.6°C. Temperatures at which the stock daphnids were kept were observed to be below the range given in the protocol of 20 ± 1°C. This small deviation was considered not to have affected the outcome or the validity of the test as there was no effect on survival and reproduction of the stock daphnids. The lighting cycle was controlled to give a 16 hours light and 8 hours darkness cycle with 20 minute dawn and dusk transition periods. Each culture was fed daily with a suspension of algae (*Chlorella* sp.). Culture conditions ensured that reproduction was by parthenogenesis. Gravid adults were isolated the day before initiation of the test, such that the young daphnids produced overnight were less than 24 hours old. These young were removed from the cultures and used for testing. The diet and diluent water are considered not to contain any contaminant that would affect the integrity or outcome of the study.

3.2 Test Water

The reconstituted water used for both the range-finding and definitive tests was the same as that used to maintain the stock animals.

The reconstituted water is defined in Appendix 3.

3.3 Procedure

3.3.1 Pre-study media preparation trial

Information supplied by the Sponsor indicated that the test material forms peroxides when in contact with air and stability analysis suggests that light conditions may accelerate peroxide formation. A media preparation trial was conducted to determine if a concentration similar to the water solubility value of 60 mg/l provided by the Sponsor could be obtained using a shake flask method.

An amount of test material (300 mg) was dispersed in 500 ml of reconstituted water and shaken at approximately 300 rpm at a temperature of 30°C for approximately 24 hours to give an initial test material dispersion of 300 mg/l. This was prepared in duplicate to allow sufficient volume for sampling. After 24 hours the shaking was stopped and the dispersions pooled prior to removal of any undissolved test material by both filtration (0.2 µm Gelman AcroCap filter, first approximate 50 and 100 ml discarded) and centrifugation for 30 minutes (10000 and 40000 *g*) to give a saturated solution.

Samples were taken from each prepared saturated solution to determine the amount of dissolved test material by Dissolved Organic Carbon (DOC) analysis. The amount of test material in solution was shown to be 97 mg/l using the shake flask method followed by filtration (first approximate 100 ml discarded) (see Appendix 4).

Given that all the results obtained were of a similar order and higher than the water solubility value of 60 mg/l supplied by the Sponsor it was considered that this method of preparation was appropriate for use in this study.

3.3.2 Range-finding test

Information supplied by the Sponsor indicated that the test material may be light sensitive therefore all test media preparations were carried out under a non-actinic light source and the test vessels were shielded from the light.

The test concentrations to be used in the definitive test were determined by a preliminary range-finding test.

In the range-finding test *Daphnia magna* were exposed to a series of nominal test concentrations of 0.010, 0.10, 1.0, 10, and 100 mg/l. The test material was dissolved directly in water.

An amount of test material (300 mg) was dispersed in 500ml of reconstituted water and shaken at approximately 300 rpm at a temperature of 30°C for approximately 24 hours. After 24 hours the shaking was stopped and the mixture allowed to cool to approximately 20°C. Any undissolved test material was removed by filtration (0.2µm Gelman AcroCap filter, first approximate 100 ml discarded) to give a saturated solution with a nominal concentration of 100 mg/l* from which serial dilutions were made to give the 10, 1.0 and 0.10 mg/l* test concentrations.

Each prepared concentration was inverted several times to ensure adequate mixing and homogeneity.

In the range-finding test 10 daphnids were placed in each test and control vessel and maintained in a temperature controlled room at approximately 20°C in the dark. Each 300 ml test and control vessel contained 300 ml of test media and was sealed to minimise test material losses. After 24 and 48 hours the number of immobilised *Daphnia magna* were recorded.

The control group was maintained under identical conditions but not exposed to the test material.

3.3.3 Definitive test

Based on the results of the range-finding test and preliminary chemical analyses, the test material solutions for the definitive test were prepared by shaking an excess (300 mg/500 ml) of test material in reconstituted water at approximately 200 rpm at a temperature of 30°C for 24 hours. After 24 hours the shaking was stopped and the dispersion was cooled to approximately 20°C and any undissolved test material was removed by filtration (0.2 µm Gelman AcroCap, first approximate 100 ml discarded) to give a saturated solution which was then further diluted, as necessary, to produce the test groups.

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* As no measured test concentration was available for the saturated solution prior to conducting the range-finding test, these test concentrations are based on the results of Dissolved Organic Carbon analysis of the saturated solution prepared during the media preparation trial.

3.3.3.1 Preparation of the test material

For the purpose of the definitive test the required amount of test material was added to each test vessel using the method described in Section 2.2.

3.3.3.2 Exposure conditions

As in the range-finding test 300 ml glass stoppered conical flasks containing approximately 300 ml of test preparation were used. At the start of the study 10 daphnids were placed in each test and control vessel at random, in the test preparations. Duplicate test vessels were used for each test and control group. The test vessels were then sealed to minimise test material losses and maintained in a temperature controlled room at approximately 20°C in the dark. The daphnids were not individually identified, received no food during exposure and the test vessels were not aerated.

The control groups was maintained under identical conditions but not exposed to the test material.

The test preparations were not renewed during the exposure period. Any immobilisation or adverse reactions to exposure were recorded at 24 and 48 hours after the start of exposure. The criterion of effect used was that *Daphnia* were considered to be immobilised if they were unable to swim for approximately 15 seconds after gentle agitation.

3.3.3.3 Physico-chemical measurements

Water temperature was recorded daily throughout the test. Dissolved oxygen concentrations and pH were recorded at the start and termination of the test. The pH and the dissolved oxygen concentration was measured using a WTW pH/Oxi 340i pH and dissolved oxygen meter and the temperature was measured using a Hanna Instruments HI 93510 digital thermometer.

3.3.3.4 Verification of test concentrations

Water samples were taken from the control (replicates R_1 – R_2 pooled) and all test groups (replicates R_1 – R_2 pooled) at 0 and 48 hours and a sample of the saturated solution was taken at 0 hours for quantitative analysis.

Duplicate samples were taken and stored at approximately -20°C for further analysis if necessary.

All sample bottles were shielded from the light.

The method of analysis, stability, recovery and test preparation analyses are described in Appendix 2.

3.3.3.5 Evaluation of data

The EC_{50} values and associated confidence limits at 24 and 48 hours and the slope of the response curve and its standard error based on time-weighted mean measured concentrations were calculated by the maximum-likelihood probit method (Finney 1971) using the ToxCalc computer software package (ToxCalc 1999).

Probit analysis is used where two or more partial responses to exposure are shown.

The time-weighted mean measured test concentrations were calculated as follows:

$$TWM = \frac{\text{Total area}}{\text{Total number of days of the test}}$$

$$\text{where Total area} = \frac{C_0 - C_1}{\ln(C_0) - \ln(C_1)} \text{ x days}$$

TWM = time-weighted mean measured test concentration (mg/l)
C_0 = measured concentration at the start of each renewal period (mg/l)
C_1 = measured concentration at the end of each renewal period (mg/l)
Days = number of days in the renewal period

3.4 Positive Control

A positive control using potassium dichromate as the reference material was conducted using test concentrations of 0.32, 0.56, 1.0, 1.8 and 3.2 mg/l (Safepharm Laboratories Project No: 0039/0874).

The required amount of reference material was added to the test vessels using the method described in Section 2.3.

Exposure conditions for the positive control were similar to those used in the definitive test.

The temperature was maintained at 20.0°C to 21.1°C. A single temperature at 0-hours was measured to be slightly in excess of the 20 ± 1°C given in the protocol. This was considered not

5. RESULTS

5.1 Pre-study Media Preparation Trial

The results of the Dissolved Organic Carbon (DOC) analysis during the media preparation trial (see Appendix 4) showed that the results obtained from both the centrifuged (10000 *g*) and filtered (first approximate 100 ml discarded) samples showed measured test concentration of 96 and 97 mg/l respectively. As the concentrations obtained were higher than the water solubility value supplied by the Sponsor and giving due regard to the fact that the test material may form peroxides when in contact with air it was considered that a saturated solution prepared by shaking for 24 hours at 30°C and 300 rpm prior to filtration through a 0.2 µm Gelman AcroCap filter (first approximate 100 ml discarded) was the most appropriate for use in this study.

5.2 Range-finding Test

Cumulative immobilisation data from the exposure of *Daphnia magna* to the test material during the range-finding test are given in Table 1.

No immobilisation was observed at the test concentrations of 0.010 mg/l. However, immobilisation was observed at 0.10, 1.0, 10 and 100 mg/l.

Based on this information time weighted mean measured test concentrations of 0.0053, 0.0080, 0.010, 0.033, 0.036, 0.065, 0.15, 0.29 and 0.58 mg/l were selected for the definitive test.

5.3 Definitive Test

5.3.1 Immobilisation data

Cumulative immobilisation data from the exposure of *Daphnia magna* to the test material during the definitive test are given in Table 2. The relationship between percentage immobilisation and concentration at 24 and 48 hours is given in Figures 1 and 2.

5.3.2 Observations on test material solubility

The test media preparations were observed to be clear colourless solutions throughout the duration of the test.

to affect the results of the test as no adverse effects of exposure were observed in the control daphnids throughout the duration of the test.

3.4.1 Evaluation of data

An estimate of the EC_{50} value at 3 hours was given by inspection of the immobilisation data.

The EC_{50} value and associated confidence limits at 24 hours and the slope of the response curve and its standard error were calculated by the maximum-likelihood probit method (Finney 1971) using the ToxCalc computer software package (ToxCalc 1999).

The EC_{50} value and associated confidence limits at 48 hours were calculated using the trimmed Spearman-Karber method (Hamilton *et al* 1977) using the ToxCalc computer software package (ToxCalc 1999).

Probit analysis is used where two or more partial responses to exposure are shown.

When only one partial response is shown the trimmed Spearman-Karber method is appropriate.

4. ARCHIVES

Unless instructed otherwise by the Sponsor, all original data and the final report will be retained in the Safepharm archives for five years, after which instructions will be sought as to further retention or disposal.

5.3.3 Physico-chemical measurements

The results of the physico-chemical measurements are given in Appendix 5. Temperature was maintained at approximately 20°C throughout the test, while there were no treatment related differences for oxygen concentration or pH.

5.3.4 Verification of test concentrations

Analysis of the saturated solution at 0 hours (see Appendix 2) showed a measured concentration of 103 mg/l.

Analysis of the test preparations at 0 hours (see Appendix 2) showed measured test concentrations to range from 0.0063 to 0.66 mg/l and at 48 hours showed measured test concentrations to range from 0.0044 to 0.51 mg/l. This decline in measured test concentrations at 48 hours was considered to be due to the volatility of the test material although every precaution was taken to minimise any losses.

Given this decline in measured test concentrations over the 48-Hour period it was considered justifiable to base the results on the time-weighted mean measured test concentrations of the test media in order to give a "worst case" analysis of the data.

Analysis of the immobilisation data by the probit method (Finney 1971) at 24 and 48 hours based on the time-weighted mean measured test concentrations gave the following results:

Time (h)	EC_{50} (mg/l)	95% Confidence limits (mg/l)
24	0.24	0.19 - 0.29
48	0.13	0.10 - 0.16

The No Observed Effect Concentrations after 24 and 48 hours exposure were 0.065 and 0.036 mg/l respectively. The No Observed Effect Concentration is based upon zero immobilisation at this concentration.

The slopes and their standard errors of the response curves at 24 and 48 hours were 4.6 (SE = 0.9) and 4.3 (SE = 0.7) respectively.

5.4 Positive Control

Cumulative immobilisation data from the exposure of *Daphnia magna* to the reference material (Safepharm Laboratories Project No: 0039/0874) during the positive control are given in Table 3. The relationship between percentage immobilisation and concentration at 24 and 48 hours is given in Figures 3 and 4.

Inspection of the immobilisation data at 3 hours and analysis of the immobilisation data by the probit method (Finney 1971) at 24 hours and the trimmed Spearman-Karber method (Hamilton *et al* (1977)) at 48 hours based on the nominal test concentrations gave the following results:

Time (h)	EC_{50} (mg/l)	95% Confidence limits (mg/l)
3	> 3.2	-
24	1.2	1.0 - 1.4
48	0.85	0.76 - 0.96

The No Observed Effect Concentration after 24 and 48 hours was 0.56 mg/l. The No Observed Effect Concentration is based upon zero immobilisation at this concentration.

The slope and standard error of the response curve at 24 hours was 9.2 (SE = 2.1). Due to the unsuitable nature of the data it was not possible to calculate the slope and standard error of the response curve at 48 hours.

The results from the positive control with potassium dichromate were within the normal range for this reference material. The mean 48-Hour EC_{50} value calculated from all positive controls was 0.82 mg/l (sd = 0.22).

6. CONCLUSION

The acute toxicity of the test material to the freshwater invertebrate *Daphnia magna* has been investigated based on the time weighted mean measured test concentrations of the test media and gave a 48-Hour EC_{50} value of 0.13 mg/l with 95% confidence limits of 0.10 - 0.16 mg/l. The No Observed Effect Concentration at 48 hours was 0.036 mg/l.

7. REFERENCES

Finney, D J (1971) Statistical Method in Biological Assay. London : Griffin and Company Ltd.

Hamilton, M A, Russo, R C and Thurston, R V (1977) Trimmed Spearman-Karber Method for Estimating Median Lethal Concentration in Toxicity Bioassays. *Environ Sci Technol* **11**, 714-719.

ToxCalc Version 5.0.23C (1999), Tidepool Scientific Software, McKinleyville, CA 95519, USA.

1,5-Cyclooctadiene (COD): ACUTE TOXICITY TO *DAPHNIA MAGNA*

Table 1 Cumulative Immobilisation Data in the Range-finding Test

Nominal Concentration (mg/l)	Cumulative Immobilised *Daphnia* (Initial Population: 10 Per Replicate)	
	24 Hours	48 Hours
Control	0	0
0.010	0	0
0.10	0	3
1.0	10	10
10	10	10
100	10	10

1,5-Cyclooctadiene (COD): ACUTE TOXICITY TO *DAPHNIA MAGNA*

Table 2 Cumulative Immobilisation Data in the Definitive Test

Time-weighted Mean Measured Concentration (mg/l)*	Cumulative Immobilised *Daphnia* (Initial Population: 10 Per Replicate)							
	24 Hours				48 Hours			
	R_1	R_2	Total	%	R_1	R_2	Total	%
Control	0	0	0	0	0	0	0	0
0.0053	0	0	0	0	0	0	0	0
0.0080	0	0	0	0	0	0	0	0
0.010	0	0	0	0	0	0	0	0
0.033	0	0	0	0	0	0	0	0
0.036	0	0	0	0	0	0	0	0
0.065	0	0	0	0	2	2	4	20
0.15	2	3	5	25	4	5	9	45
0.29	5	6	11	55	10	10	20	100
0.58	10	10	20	100	10	10	20	100

$R_1 - R_2$ = Replicates 1 and 2
* Time-weighted mean measured test concentrations at the end of the test

1,5-Cyclooctadiene (COD): ACUTE TOXICITY TO *DAPHNIA MAGNA*

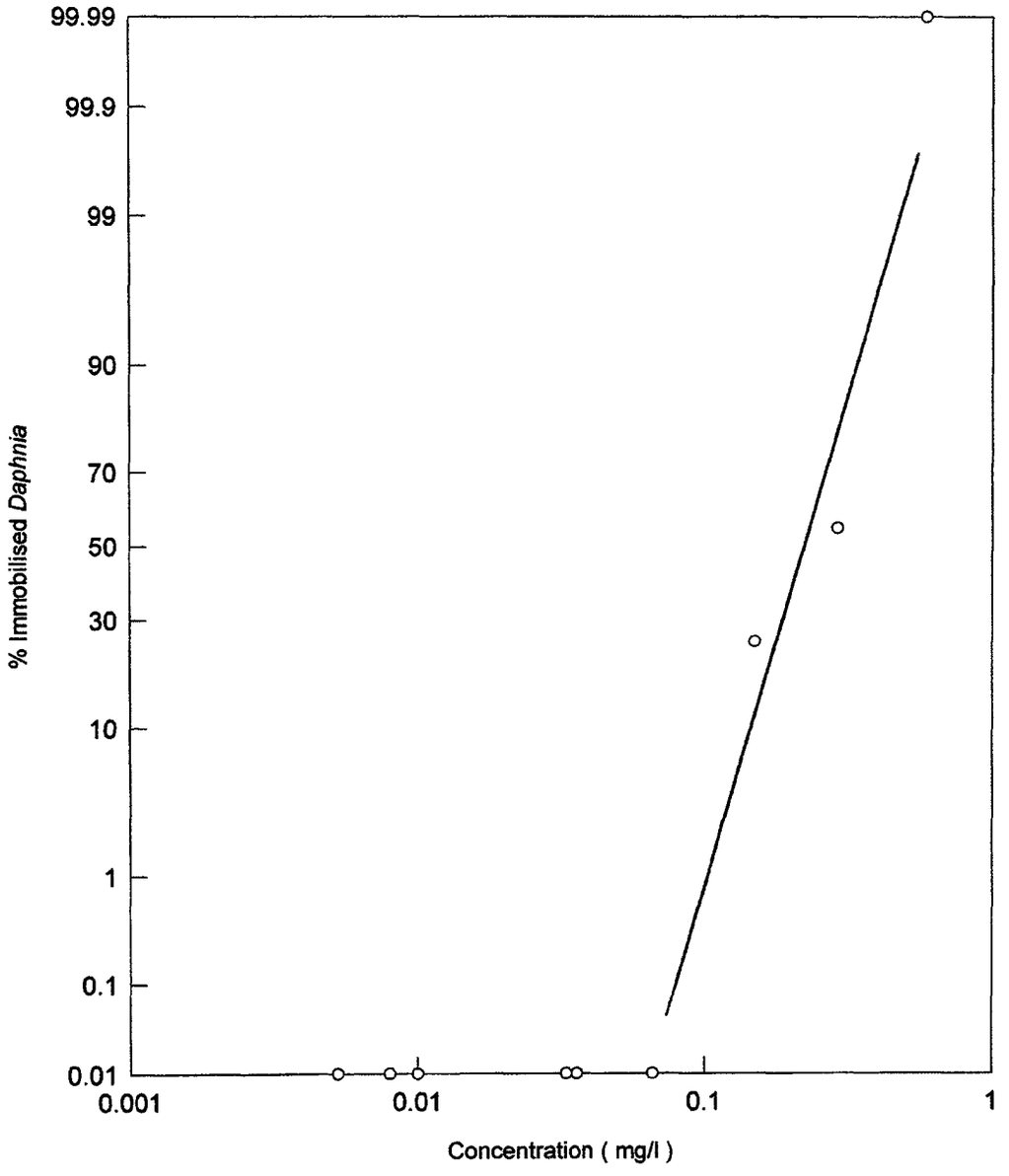
Table 3 Cumulative Immobilisation Data in the Positive Control

Nominal Concentration (mg/l)	Cumulative Immobilised *Daphnia* (Initial Population: 10 Per Replicate)											
	3 Hours				24 Hours				48 Hours			
	R_1	R_2	Total	%	R_1	R_2	Total	%	R_1	R_2	Total	%
Control	0	0	0	0	0	0	0	0	0	0	0	0
0.32	0	0	0	0	0	0	0	0	0	1*	1	5
0.56	0	0	0	0	0	0	0	0	0	0	0	0
1.0	0	0	0	0	3	2	5	25	9	6	15	75
1.8	0	0	0	0	9	10	19	95	10	10	20	100
3.2	0	0	0	0	10	10	20	100	10	10	20	100

$R_1 - R_2$ = Replicates 1 and 2
* Immobilisation considered to be due to natural causes rather than a toxic effect as no immobilisation was observed at the 0.56 mg/l test concentration

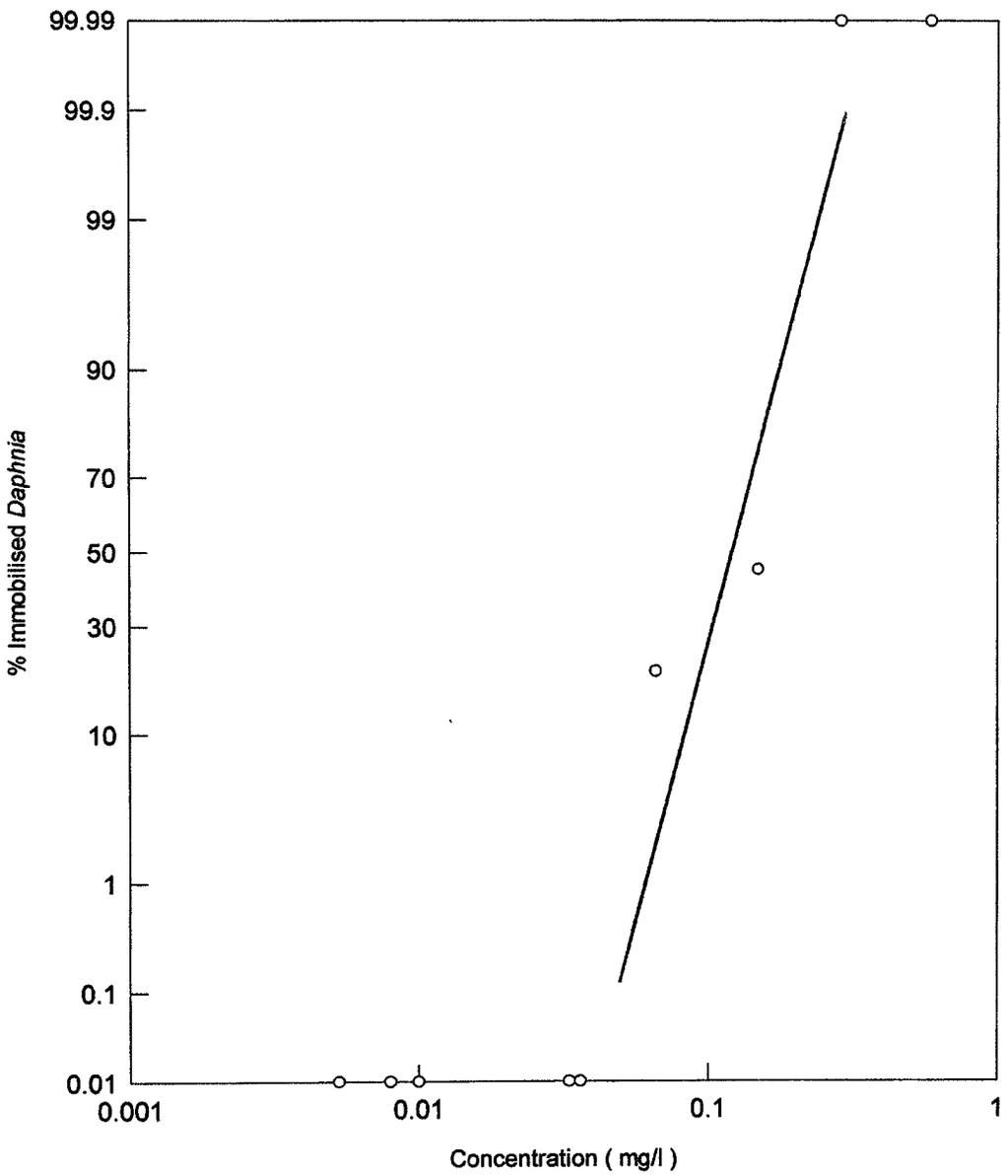
1,5-Cyclooctadiene (COD): ACUTE TOXICITY TO *DAPHNIA MAGNA*

Figure 1 **Concentration-Response Curve After 24 Hours (Based On Time-weighted Mean Measured Concentrations)**



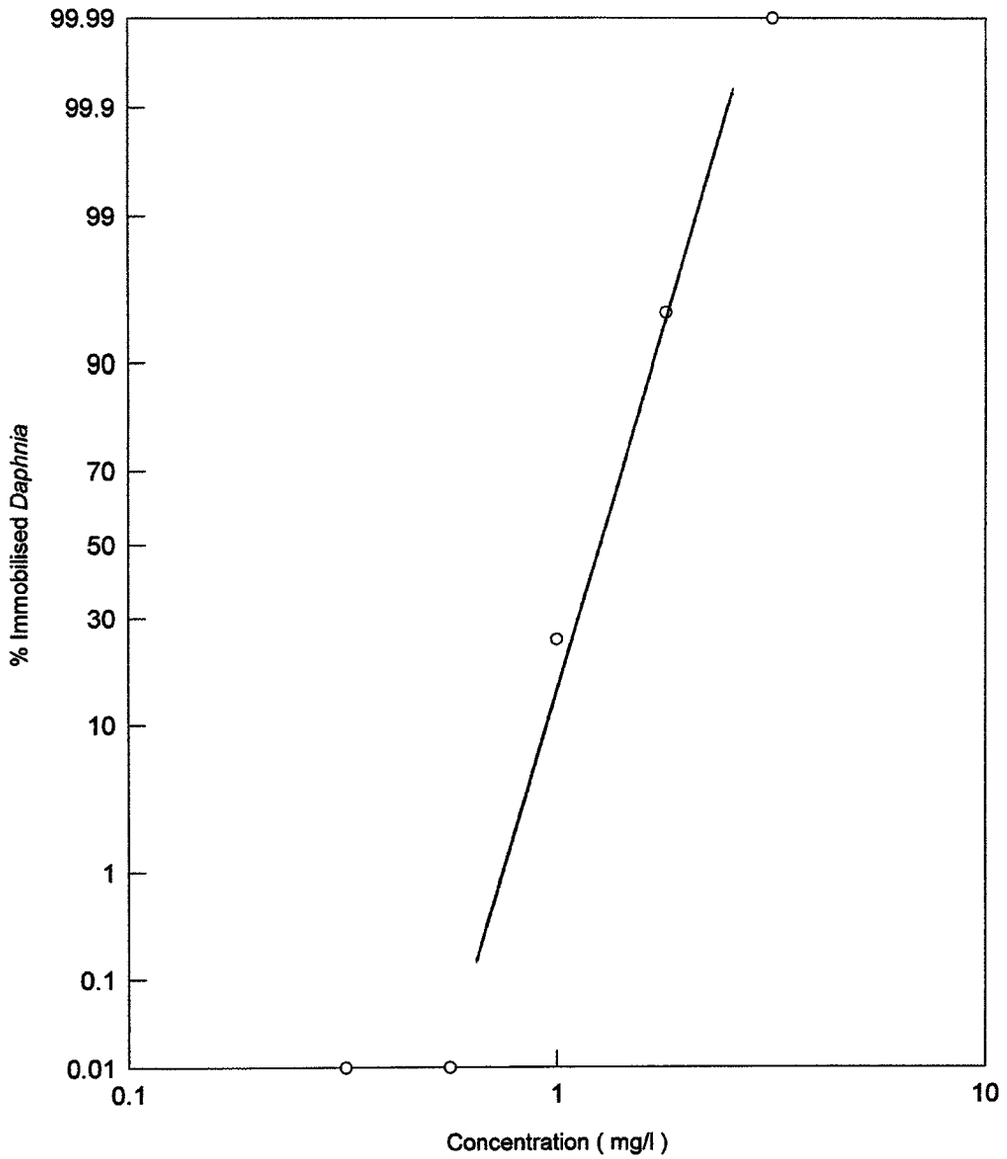
1,5-Cyclooctadiene (COD): ACUTE TOXICITY TO *DAPHNIA MAGNA*

Figure 2 **Concentration-Response Curve After 48 Hours (Based On Time-weighted Mean Measured Concentrations)**



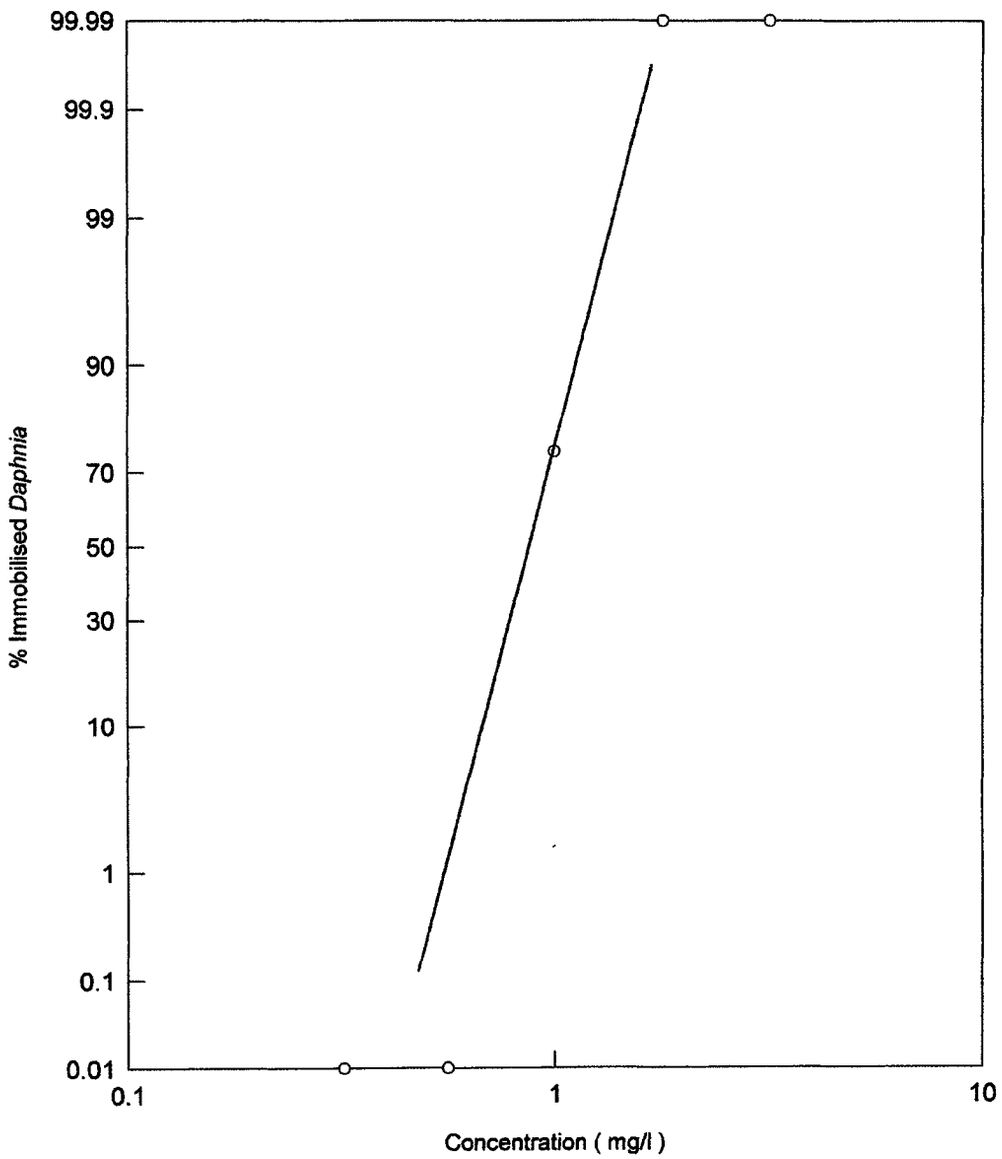
1,5-Cyclooctadiene (COD): ACUTE TOXICITY TO *DAPHNIA MAGNA*

Figure 3 Concentration-Response Curve After 24 Hours in the Positive Control



1,5-Cyclooctadiene (COD): ACUTE TOXICITY TO *DAPHNIA MAGNA*

Figure 4 Concentration-Response Curve After 48 Hours in the Positive Control



1,5-Cyclooctadiene (COD): ACUTE TOXICITY TO *DAPHNIA MAGNA*

Appendix 1 Protocol

SafePharm Laboratories

PROTOCOL

TEST MATERIAL	:	1,5-Cyclooctadiene (COD)
STUDY TYPE	:	Acute Toxicity to *Daphnia magna* (OECD (2004) 202, EEC C.2)
TEST METHOD	:	900.16
PROJECT NUMBER	:	2231/0009
PROPOSED START DATE	:	September 2006
PROPOSED COMPLETION DATE	:	October 2006
TARGET (DRAFT) REPORT DATE	:	November 2006
SPONSOR	:	INVISTA S.a.r.l. INVISTA Building 4123 East 37th Street North Wichita KS 67201 UNITED STATES OF AMERICA
SPECIAL CONDITIONS	:	The test will be conducted in a closed system with no headspace. The results will be calculated on measured as well as nominal test concentrations.

APPROVED FOR SPONSOR BY: ..... DATE: 12/06/06

AUTHORISED BY: ..... DATE: 15 JUN 2006
T J Goodband BSc
STUDY DIRECTOR

This protocol is issued without signature by the Study Director to enable changes to be made if necessary prior to authorisation. Sponsors should sign and return the document to indicate approval and GLP authorisation will be confirmed by the Study Director's signature prior to the start of the study.

1,5-Cyclooctadiene (COD): ACUTE TOXICITY TO *DAPHNIA MAGNA*

Appendix 1 Protocol (Continued)

ACUTE TOXICITY TO *DAPHNIA MAGNA*

1. INTRODUCTION AND OBJECTIVES

This test method details a study designed to comply with the following regulatory guidelines:

i) The OECD Guidelines for Testing of Chemicals (2004) No 202 "*Daphnia* sp., Acute Immobilisation Test" referenced as Method C.2 of Commission Directive 92/69/EEC (which constitutes Annex V of Council Directive 67/548/EEC).

The purpose of this study is to assess the acute toxicity of the test material to the freshwater invertebrate *Daphnia magna*. The toxicity will be expressed as the median effect concentration (EC_{50}) or median effect loading rate (EL_{50}) for immobilisation.

The No Observed Effect Concentration (NOEC) or No Observed Effect Loading rate (NOEL) will also be determined.

Daphnia magna is a freshwater invertebrate representative of a wide variety of natural habitats, and can therefore be considered as an important non-target organism in freshwater ecosystems.

The dispersal of the test material in the surrounding medium is considered to represent the most probable route of exposure in the environment.

The work described will be performed in compliance with UK GLP standards (Schedule 1, Good Laboratory Practice Regulations 1999 (SI 1999/3106 as amended by SI 2004/0994)). These Regulations are in accordance with GLP standards published as OECD Principles on Good Laboratory Practice (revised 1997, ENV/MC/CHEM(98)17); and are in accordance with, and implement, the requirements of Directives 2004/9/EC and 2004/10/EC.

These international standards are acceptable to the Regulatory agencies of the following countries: Australia, Austria, Belgium, Canada, the Czech Republic, Denmark, Finland, France, Germany, Greece, Hungary, Iceland, Ireland, Israel, Italy, Japan, Republic of Korea, Luxembourg, Mexico, The Netherlands, New Zealand, Norway, Poland, Portugal, Slovenia, South Africa, Spain, Sweden, Switzerland, Turkey, the United Kingdom, and the United States of America.

1,5-Cyclooctadiene (COD): ACUTE TOXICITY TO *DAPHNIA MAGNA*

Appendix 1 Protocol (Continued)

2. TEST FACILITY

Safepharm Laboratories Ltd
Shardlow Business Park
Shardlow
Derbyshire
DE72 2GD

3. ANIMALS

Specification

Daphnia magna of the 1st instar derived from in-house laboratory cultures.

Justification

Daphnia magna has been selected following the recommendations of OECD Guideline No 202 and EEC Directive 92/69/EEC.

4. ANIMAL HUSBANDRY

Environment

Water Temperature:	20 ± 1°C
Lighting:	Sixteen hours of continuous artificial light and eight hours continuous darkness with 20 minute dawn and dusk transition periods.

Housing

Animals will be maintained in 2-3 litre polypropylene beakers, stocked at a density of 30-80 daphnids per vessel initially.

Water and Diet

The animals will be maintained in a reconstituted water as defined in Appendix 1. The daphnids will be fed an algal suspension details of which will be documented in the data. Any change in species of algae

Appendix 1 Protocol (Continued)

fed to the daphnids will be documented in the data. Culture conditions ensure that reproduction will be by parthenogenesis. Cultures containing gravid adults will have any young daphnids removed at a time during the day prior to initiation of the test such that the young daphnids produced overnight will be equal to or less than 24 hours old. The diet and diluent water are considered not to contain any contaminant that would affect the integrity or outcome of the study.

5. PRE-TEST PROCEDURES

Identification

The animals will not be individually identified.

Allocation

Animals will be selected at random from the total number collected. No other method of randomisation will be used.

6. TEST MATERIAL AND EXPERIMENTAL PREPARATION

Identification

Supplied by the study Sponsor with the details of hazardous properties if known. The integrity of supplied data relating to the identity, purity and stability of the test material will be the responsibility of the Sponsor.

Storage

Room temperature, in the dark, unless otherwise specified by the Sponsor.

Preparation

The test material will be dissolved/dispersed in the test water once at the start of the study (static test) or on a daily (semi-static test) or continuous (dynamic test) basis if deemed necessary. If auxiliary solvents or surfactants are used to aid in the dispersion, they will be used at a maximum concentration of 100 µl/l (final volume). Other methods such as prolonged mixing, ultrasonication, high shear force stirring or the

Appendix 1 Protocol (Continued)

production of Water Accommodated Fractions (WAFs) or saturated solutions may be used depending on the nature of the test material. Details of the method of preparation will be documented in the data.

Analysis

Details of identification of the test material will be supplied by the study Sponsor. The test material formulations will be analysed for concentration and stability by Safepharm Analytical Services.

Absorption

Absorption is via the membranes exposed to the surrounding water. Specific determination of absorption will not be made in this study.

7. STUDY DESIGN

Administration

The test material will normally be dissolved/dispersed in the test water once at the start of the study. However if necessary the test material may be dissolved/dispersed in the test water on a daily or continuous basis. The study duration will be 48 hours. The actual study dates will be documented in the data.

Test Vessels

Glass jars (250 ml) containing approximately 200 ml or 250 ml of test solution, covered with aluminium foil to reduce evaporation. Volatile chemicals will be tested in completely filled and stoppered vessels containing approximately 300 ml of test solution.

For static and semi-static tests the test vessels will be allocated to a position in the relevant area in the laboratory in a random fashion.

For dynamic tests randomisation of the test vessels will not be performed due to the complexity of the dosing system.

1,5-Cyclooctadiene (COD): ACUTE TOXICITY TO *DAPHNIA MAGNA*

Appendix 1 Protocol (Continued)

Loading

Ten 1st instar daphnids per vessel. Twenty animals will be used per concentration divided into two batches of ten.

Test Concentrations

Five to nine, logarithmically spaced by a factor not exceeding 2.2, test groups plus 1 control and 1 solvent control if appropriate, each in duplicate, will be employed in the test. The use of nine test concentrations enables calculation of both a 24-Hour and 48-Hour EC_{50} (or EL_{50}) value. The actual test concentrations are assigned according to preliminary range-finding tests, and will be documented in the data prior to the start of the main study.

Where no effect is observed in the range-finding test at either 100 mg/l or the maximum limit of water solubility then a "Limit Test" will be carried out using 4 replicate test vessels of five daphnids at that concentration together with a control and solvent control, if appropriate, each of 4 replicates of five daphnids.

Where studies are conducted using Water Accommodated Fractions (WAFs) or saturated solutions, test loading rates in excess of 100 mg/l may be used.

The test media will not be renewed unless specified otherwise.

If the test media is renewed on a daily basis (semi-static test) the *Daphnia* will be transferred to the fresh media by wide-bore pipette.

The control group will be exposed to the test water alone. Data from the control group may be shared with similar concurrent studies.

Where auxiliary solvents/surfactants are used, a second control will be employed. The daphnids will be exposed to a concentration of solvent equal to that employed in the highest concentration in the test series.

The test will be carried out without adjustment of pH. If the pH falls outside the range 6-9, the Sponsor and the Competent Authorities will be contacted for advice on whether a second test should be performed with adjustment of pH to that of the dilution water. However, pH adjustment will not be carried out if the test solutions are changed to any significant extent and if a chemical reaction or precipitation is caused.

A positive control study will be conducted approximately every six months using potassium dichromate.

1,5-Cyclooctadiene (COD): ACUTE TOXICITY TO *DAPHNIA MAGNA*

Appendix 1 Protocol (Continued)

Environment and Feeding

The environmental parameters will be as used to maintain the stock animals. No auxiliary aeration will be supplied to the test vessels. The oxygen concentration should remain ≥ 60% ASV (Air Saturation Value) at the end of the test. However, during normal working practice the oxygen concentration is maintained at ≥ 60% Air Saturation Value (ASV) (≥ 5.3 mg O_2/l). The animals will not be fed during exposure.

Verification of Test Concentrations

Samples of the test media will normally be taken at 0 and 48 hours and analysed. However, should testing be conducted on a semi-static or continuous basis, then samples may also be taken at 24 hours (old and fresh media for semi-static tests). Where possible duplicate samples will be taken at each occasion and stored at approximately -20°C, or other methods appropriate to the particular test material. These samples may also be analysed should the need arise. Stability analysis will also be performed to determine whether the test material is stable in water over the exposure period at the test concentrations employed. For an EC_{50} (or EL_{50}) study, samples of the control (or solvent control where applicable) and alternate concentrations will be sampled at 0 and 48 hours. However, if the stability analysis shows the test material to be unstable over the test period then all test groups will be analysed. For a "Limit Test" two samples (Replicates R_1 and R_2 pooled and R_3 and R_4 pooled) will be taken and analysed at the concentration employed.

Stock solutions may also be analysed, if necessary, for example if the test concentrations are below the limit of quantitation of the analytical method or for reasons of stability.

Final details of sample size, etc, will be documented in the data.

8. OBSERVATIONS

Any abnormal behaviour or appearance of the daphnids (sub lethal effects other than immobility) will be recorded at 24 and 48 hours.

The number of *Daphnia* which are unable to swim for approximately 15 seconds after gentle agitation of each vessel is recorded after 24 and 48 hours. For semi-static tests these observations will be performed prior to the media renewal.

For static and dynamic flow tests the temperature is recorded daily and pH and dissolved oxygen measurements are made for each vessel at the start and the end of the study. For semi-static tests,

1,5-Cyclooctadiene (COD): ACUTE TOXICITY TO *DAPHNIA MAGNA*

Appendix 1 Protocol (Continued)

measurements of pH, dissolved oxygen and temperature are made for each vessel at the start, prior to and post test media renewal and at the end of the study.

Observations will be made on the test material preparations (if necessary) during the exposure period for reasons such as settlement of test material.

9. EVALUATION OF DATA

The 24 and 48-Hour EC_{50} (or EL_{50}) (immobilisation) values and associated 95% confidence limits and slopes of the dose response curves and their standard errors (subject to the suitability of the data) will be calculated using the trimmed Spearman-Karber method of Hamilton *et al* (1977) *Environ. Sci. Technol.* **11**, 714-719, the Probit method of Finney (1971) Statistical Method in Biological Assay. London : Griffin and Company Ltd or any other appropriate method. All EC_{50} (or EL_{50}) values will be rounded to two significant figures.

The No Observed Effect Concentration (NOEC) or No Observed Effect Loading rate (NOEL) is the test concentration or loading rate where no significant immobilisation or sub-lethal effects of exposure are observed.

10. VALIDATION CRITERIA

The test is considered invalid if ≥ 10% of the control daphnids appear to be immobilised or stressed after 48 hours or if the oxygen concentration of the control vessels falls below 60% of Air Saturation Values (ASV) at the end of the test.

11. QUALITY ASSURANCE

This standard test method will be reviewed for GLP compliance and the final report will be audited by Safepharm Quality Assurance Unit. This type of study is subject to process-based QA inspection designed to encompass the major phases once per month.

12. PROTOCOL AMENDMENTS

Amendments to protocol will be made only by completion of an Amendment to Protocol form authorised by the Study Director.

Appendix 1 Protocol (Continued)

13. REPORT

The Sponsor will be informed immediately of all relevant findings. A full report containing a description of the experimental procedure, summary of observations and a discussion of the results will be prepared.

Details of the analytical method and solution analysis will be given as an Appendix.

If required a draft report will be sent to the Sponsor for review and comments before issue of the final report.

14. ARCHIVE

Unless instructed otherwise by the Sponsor, all original data and the final report will be retained in the Safepharm archives for five years after which instructions will be sought as to further retention or disposal. Further retention or return will be chargeable to the Sponsor.

1,5-Cyclooctadiene (COD): ACUTE TOXICITY TO *DAPHNIA MAGNA*

Appendix 1 Protocol (Continued)

Appendix 1 Reconstituted Water

i) Stock Solutions

a)	$CaCl_2.2H_2O$	11.76 g/l
b)	$MgSO_4.7H_2O$	4.93 g/l
c)	$NaHCO_3$	2.59 g/l
d)	KCl	0.23 g/l

ii) Preparation

All chemicals must be of analytical grade. The water will be deionised water with a conductivity of $< 5\mu S\ cm^{-1}$.

Mix 25 ml of each of the four stock solutions and make up to 1 litre with water. The pH should be 7.8 ± 0.2. If necessary adjust the pH with NaOH or HCl. Aerate until the dissolved oxygen concentration is approximately the air-saturation value.

The reconstituted water has an approximate theoretical total hardness of 250 mg/l as $CaCO_3$.

Appendix 2 Verification of Test Concentrations

1. METHOD OF ANALYSIS

1.1 Introduction

The test material concentration in the test samples was determined by gas chromatography (GC) using an external standard. The test material gave a chromatographic profile consisting of a single peak.

The method was developed by the Department of Analytical Services, Safepharm Laboratories Limited.

1.2 Sample Preparation

A volume (500 ml) of test sample was extracted with dichloromethane (1 x 50 ml). An aliquot of the lower dichloromethane phase was vialled for analysis to give a final theoretical concentration of 0.10 to 10 mg/l.

1.3 Standards

Standard solutions of test material were prepared in dichloromethane at a nominal concentration of 1.0 mg/l.

1.4 Procedure

The standards and samples were analysed by GC using the following conditions:

GC System	:	Agilent Technologies 5890 incorporating autosampler and workstation
Column	:	DB-1 (30 m x 0.53 mm id, 5 µm film)
Oven temperature program	:	initial 70°C for 2 minutes rate 1 7°C/minute final 100°C for 2 minutes
Injector temperature	:	230°C

Appendix 2 Verification of Test Concentrations (Continued)

Detector temperature	:	250°C
Carrier gas and pressure	:	nitrogen at approximately 10 psi
Injection volume	:	1 µl
Injection mode	:	splitless purge on at 1 minute
Detector	:	flame ionisation detector (FID)
Retention time	:	approximately 7 minutes

2. VALIDATION

2.1 Linearity

A range of standard solutions covering 0.11 to 277 mg/l (110% of the lowest working sample concentration to 2800% of the highest working sample concentration) was analysed.

Linearity was confirmed ($R^2 = 1$) in the range from 0 to 277 mg/l.

The results are presented graphically on page 38.

2.2 Recoveries

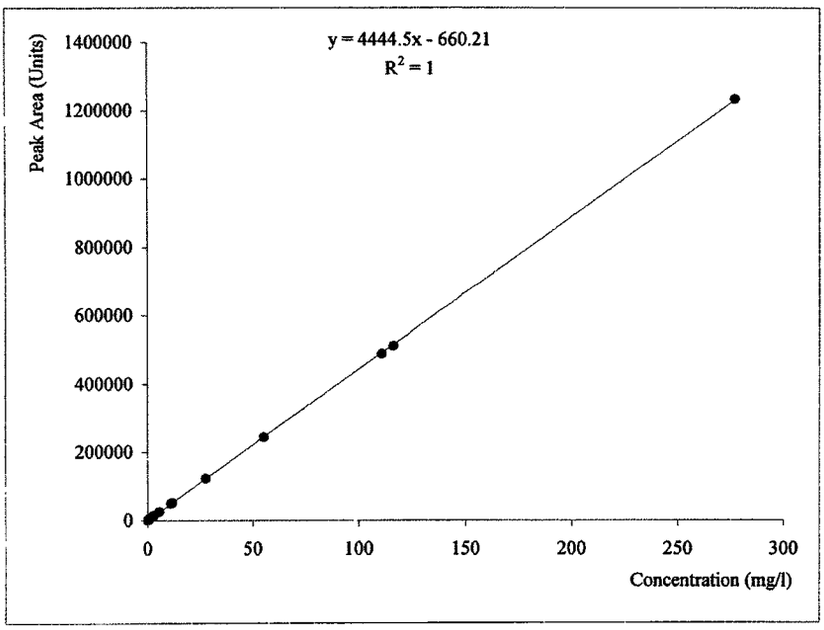
A range of preliminary test samples, accurately fortified at known concentrations of test material, was prepared and analysed.

The recovery samples were prepared by addition of a standard solution of test material to a sample of test medium. A standard solution was accurately prepared by dissolving the test material in acetone. An accurate volume of the standard solution was added to a known volume of test medium to achieve the required concentration of test material in water.

1,5-Cyclooctadiene (COD): ACUTE TOXICITY TO *DAPHNIA MAGNA*

Appendix 2 Verification of Test Concentrations (Continued)

Linearity of Detector Response



Appendix 2 Verification of Test Concentrations (Continued)

Fortification (mg/l)	Recoveries		
	(mg/l)	(%)	Mean %
0.0107	0.0112	105	100
0.0107	0.0102	95	
0.107	0.105	98	95
0.107	0.0981	91	
1.07	0.947	88	86
1.07	0.900	84	

The method has been considered to be sufficiently accurate and precise for the purposes of this test. The test sample results have not been corrected for recovery.

2.3 Limit of Quantitation

The limit of quantitation has been assessed down to 0.0056 mg/l.

3. STABILITY

A range of preliminary test samples was prepared, analysed initially and then after storage in sealed glass vessels at ambient temperature in light and dark conditions for approximately 48 hours (equivalent to the test exposure period). In addition test samples were tested for stability without prior mixing (sonication) the test sample bottles to assess for losses due to adsorption and/or insolubility and also tested for losses due to volatility.

1,5-Cyclooctadiene (COD): ACUTE TOXICITY TO *DAPHNIA MAGNA*

Appendix 2 Verification of Test Concentrations (Continued)

Nominal concentration (mg/l)	0.010	0.10	1.0
Concentration found initially (mg/l)	0.0107	0.102	0.924
Concentration found after storage in light conditions (mg/l)	0.00998	0.0959	0.927
Expressed as a percent of the initial concentration	93	94	100
Concentration found after storage in dark conditions (mg/l)	0.0113	0.0937	0.898
Expressed as a percent of the initial concentration	106	92	97
Concentration found after storage in dark conditions (mg/l) – unsonicated sample	0.0107	0.0888	0.868
Expressed as a percent of the initial concentration	100	87	94
Concentration found after storage in dark conditions (mg/l) – open vessel sample	0.00693	0.0586	0.581
Expressed as a percent of the initial concentration	65	57	63

The test samples have been shown to be stable in the test medium in light and dark conditions.

The results of the unsonicated stability vessel showed no evidence of insolubility or adherence to glass.

The results of the open vessel showed evidence of volatility.

1,5-Cyclooctadiene (COD): ACUTE TOXICITY TO *DAPHNIA MAGNA*

Appendix 2 Verification of Test Concentrations (Continued)

4. RESULTS

Sample	Time-weighted Mean Measured Concentration (mg/l)*	Concentration Found (mg/l)
0 Hours	Control	<LOQ
	0.0053	0.00633
	0.0080	0.00983
	0.010	0.0124
	0.033	0.0379
	0.036	0.0405
	0.065	0.0771
	0.15	0.169
	0.29	0.333
	0.58	0.662
	Saturated Solution	103
48 Hours	Control	<LOQ
	0.0053	0.00437
	0.0080	0.00634
	0.010	0.00819
	0.033	0.0279
	0.036	0.0317
	0.065	0.0550
	0.15	0.125
	0.329	0.257
	0.58	0.509

5. DISCUSSION

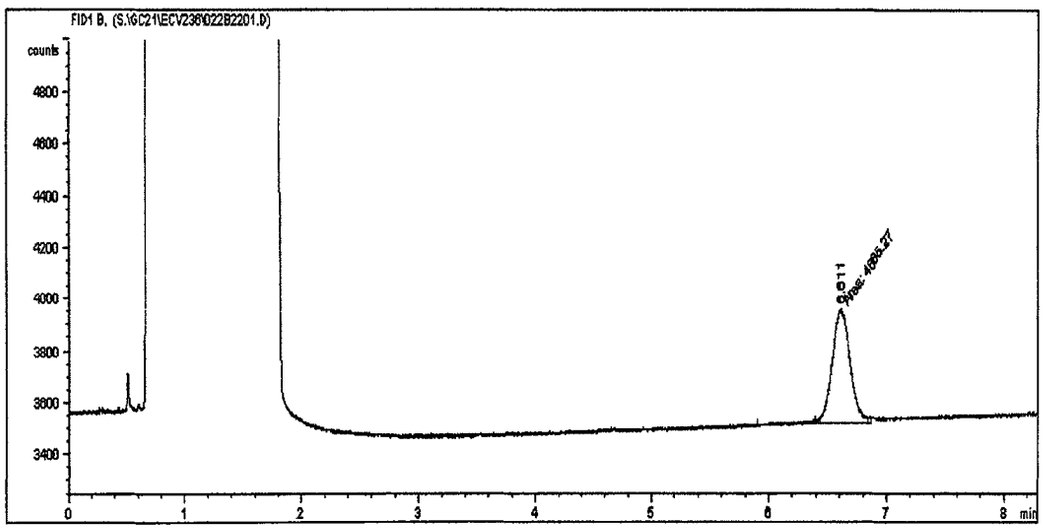
The detection system was found to have acceptable linearity. The analytical procedure had acceptable recoveries of test material in test medium. A method of analysis was validated and proven to be suitable for use.

LOQ = Limit of quantitation
* Time-weighted mean measured test concentrations at the end of the test

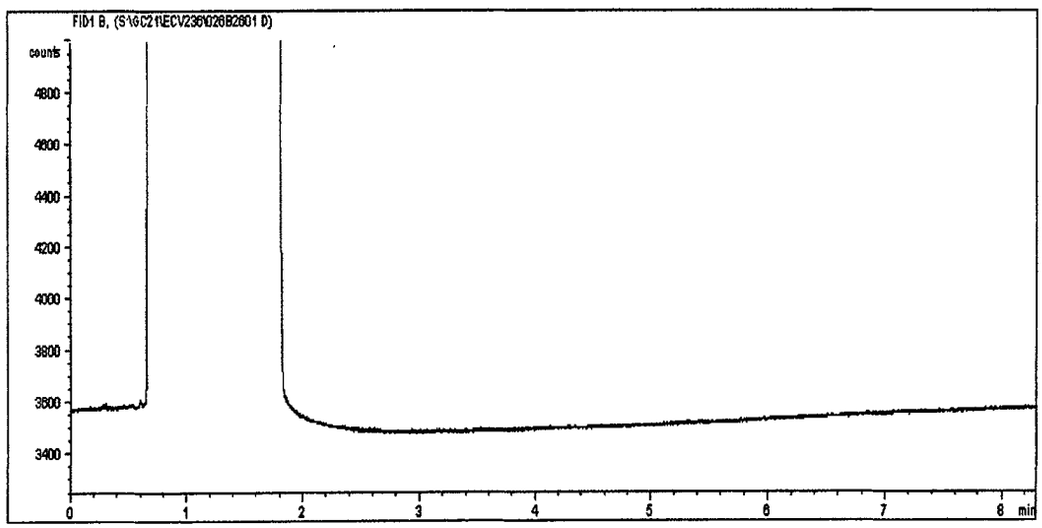
1,5-Cyclooctadiene (COD): ACUTE TOXICITY TO *DAPHNIA MAGNA*

Appendix 2 Verification of Test Concentrations (Continued)

6. TYPICAL CHROMATOGRAPHY



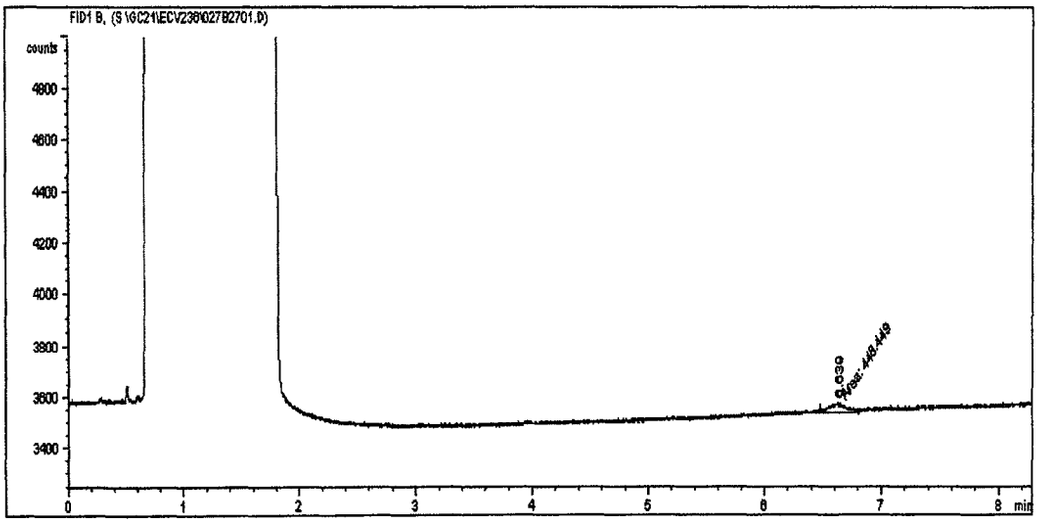
Standard 1.0 mg/l



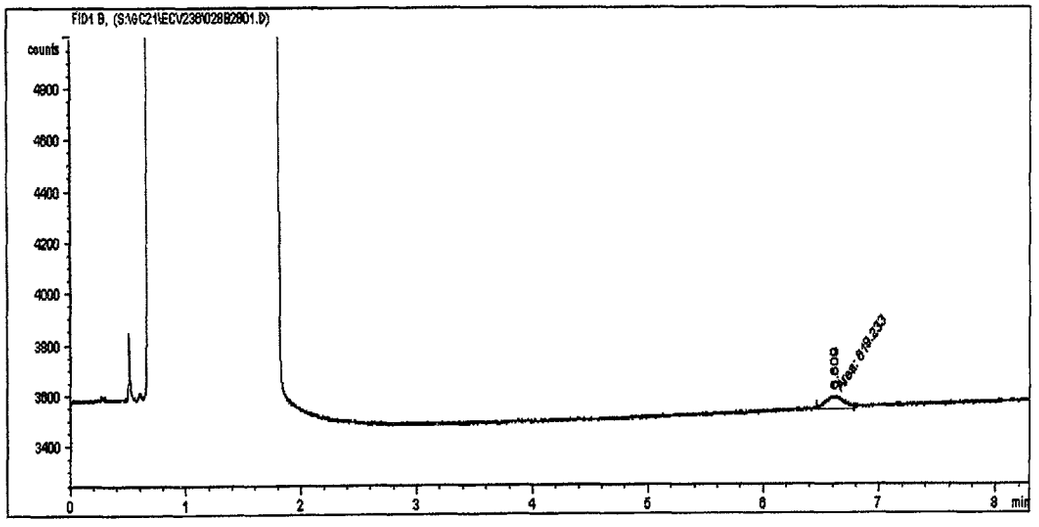
Control Sample

1,5-Cyclooctadiene (COD): ACUTE TOXICITY TO *DAPHNIA MAGNA*

Appendix 2 Verification of Test Concentrations (Continued)



Test Sample 0.0053 mg/l*



Test Sample 0.0080 mg/l*

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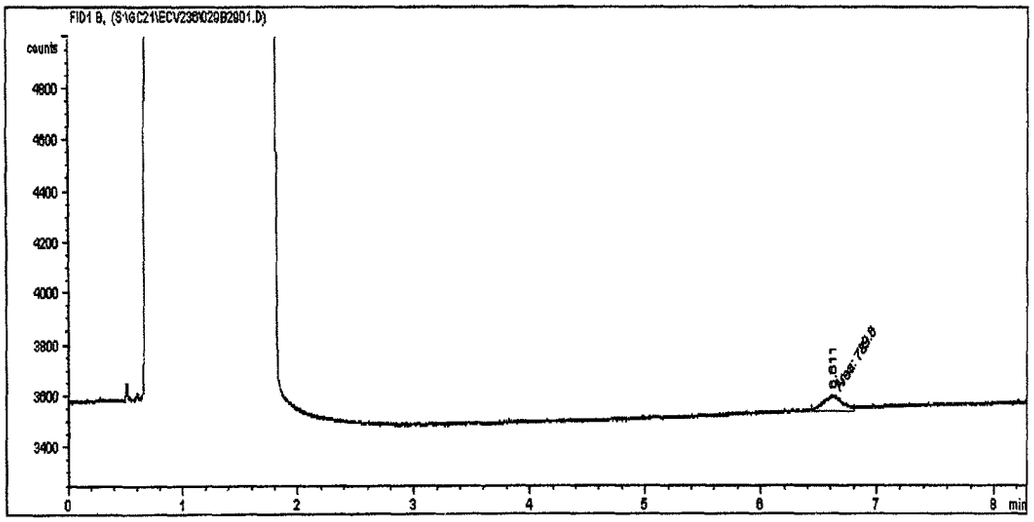
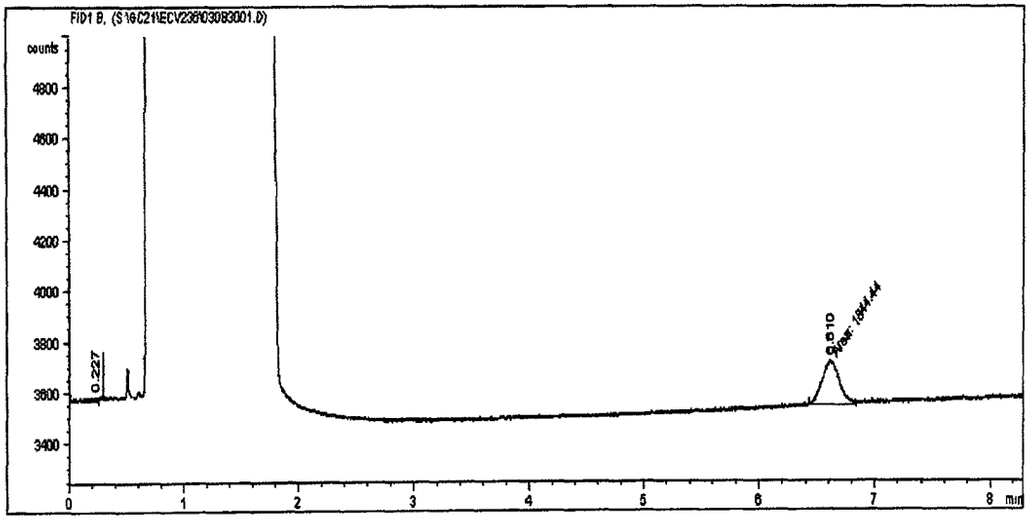
* Time-weighted mean measured test concentrations at the end of the test.

1,5-Cyclooctadiene (COD): ACUTE TOXICITY TO *DAPHNIA MAGNA*

Appendix 2 Verification of Test Concentrations (Continued)



Test Sample 0.010 mg/l*



Test Sample 0.033 mg/l*

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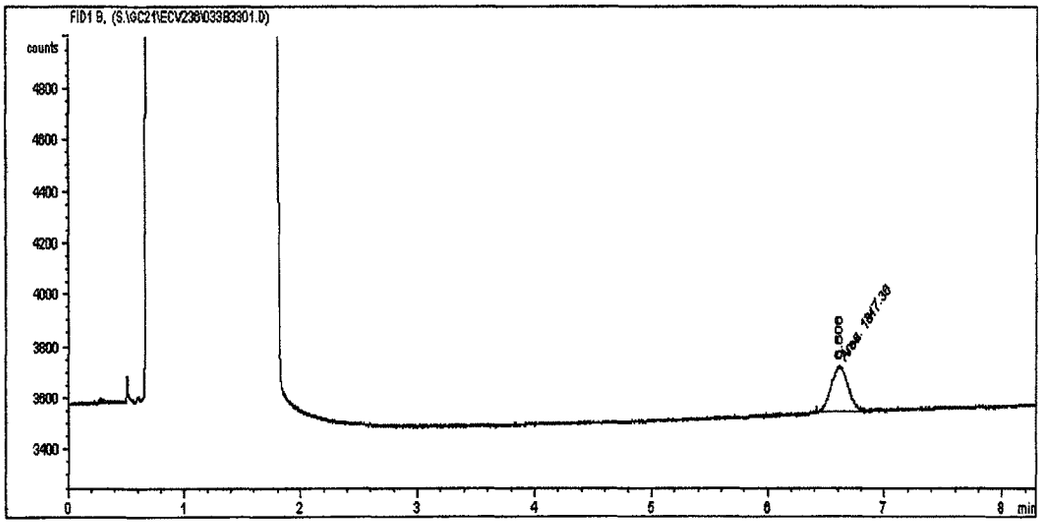
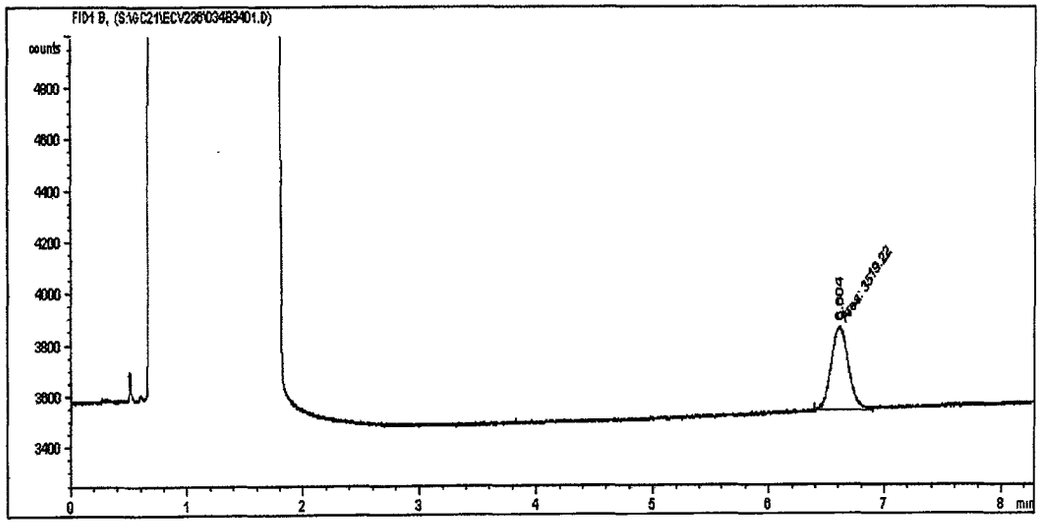
* Time-weighted mean measured test concentrations at the end of the test.

1,5-Cyclooctadiene (COD): ACUTE TOXICITY TO *DAPHNIA MAGNA*

Appendix 2 Verification of Test Concentrations (Continued)



Test Sample 0.036 mg/l*



Test Sample 0.065 mg/l*

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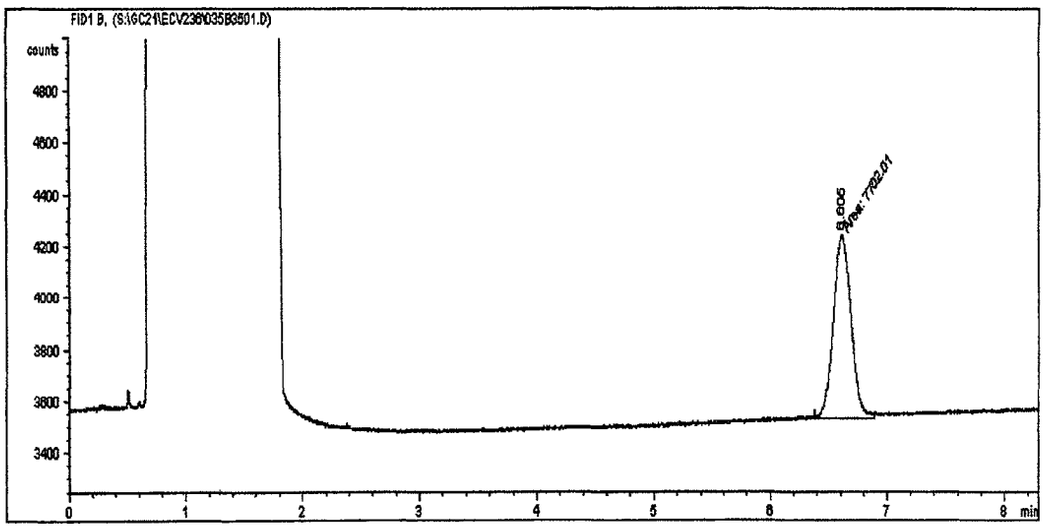
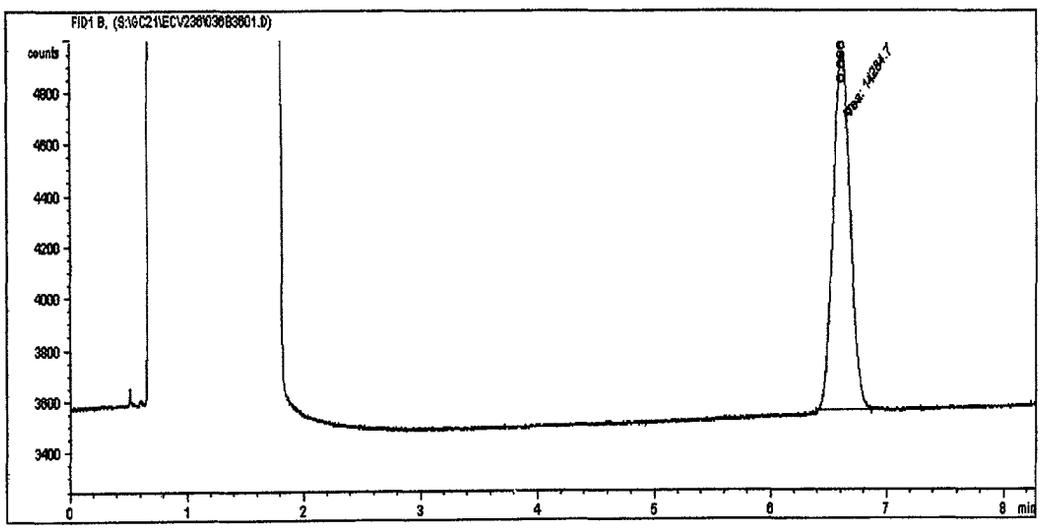
* Time-weighted mean measured test concentrations at the end of the test.

1,5-Cyclooctadiene (COD): ACUTE TOXICITY TO *DAPHNIA MAGNA*

Appendix 2 Verification of Test Concentrations (Continued)



Test Sample 0.15 mg/l*



Test Sample 0.29 mg/l*

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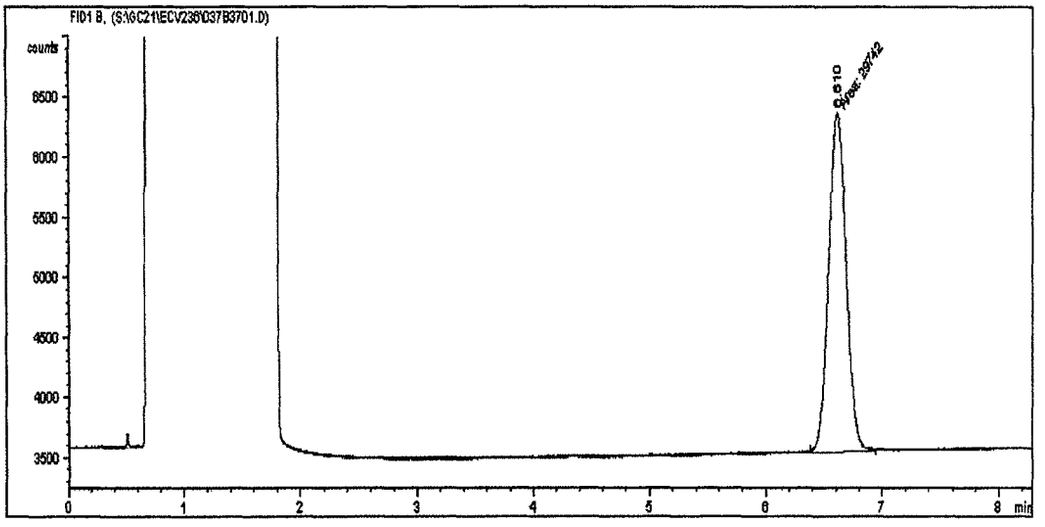
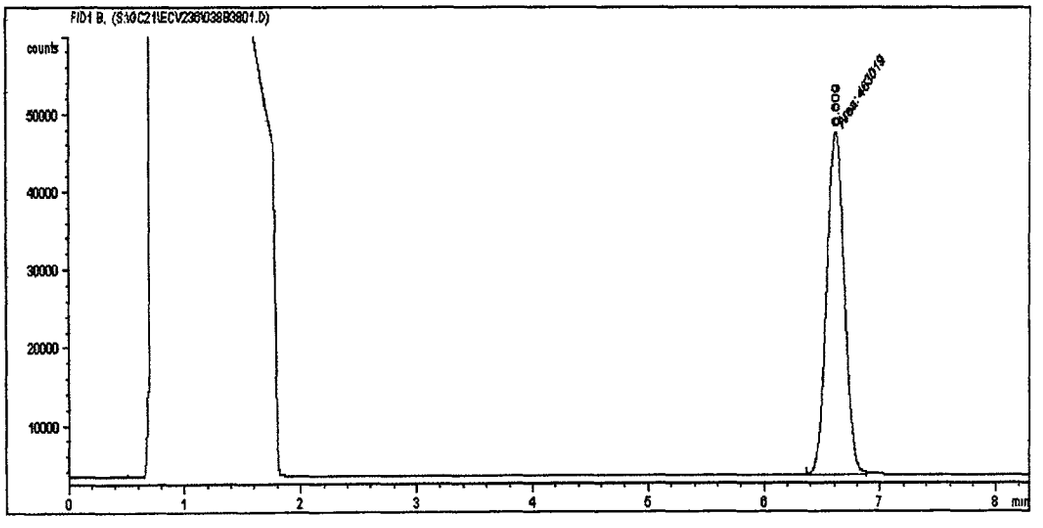
* Time-weighted mean measured test concentrations at the end of the test.

1,5-Cyclooctadiene (COD): ACUTE TOXICITY TO *DAPHNIA MAGNA*

Appendix 2 Verification of Test Concentrations (Continued)



Test Sample 0.58 mg/l*



Saturated Solution Sample

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* Time-weighted mean measured test concentrations at the end of the test.

Appendix 3 Reconstituted Water

i) Stock Solutions

a) $CaCl_2.2H_2O$	11.76 g/l
b) $MgSO_4.7H_2O$	4.93 g/l
c) $NaHCO_3$	2.59 g/l
d) KCl	0.23 g/l

ii) Preparation

An aliquot (25 ml) of each of solutions a-d was added to each litre (final volume) of deionised water with a conductivity of <5 μS cm^{-1}. The reconstituted water had a pH of 7.8 ± 0.2 adjusted (if necessary) with NaOH or HCl and was aerated until the dissolved oxygen concentration was approximately air-saturation value.

The reconstituted water had an approximate theoretical total hardness of 250 mg/l as $CaCO_3$.

Appendix 4 Pre-study Media Preparation Trial

Pre-study investigational work was carried out to determine the amount of test material that could be obtained from a shake flask method of preparation. An amount of test material (300 mg) was dispersed in 500 ml of reconstituted water and shaken at approximately 300 rpm at a temperature of 30°C for approximately 24 hours to give an initial test material dispersion of 300 mg/l. This was carried out in duplicate to allow enough media for sampling. After 24 hours the shaking was stopped for each and pooled prior to any undissolved test material was removed by filtration (0.2 µm Gelman AcroCap filter, first approximate 50 and 100 ml discarded) and centrifugation (10000 and 40000 *g*) to give saturated solutions. Samples were taken from each prepared saturated solution to determine the amount of dissolved test material by Dissolved Organic Carbon (DOC) analysis.

The results are summarised as follows:

Initial Test Material Dispersion (mg/l)	Time (Hours)		
	24		
	mg C/l	mg C/l Corrected for Control	Equivalent Test Material Concentration (mg/l)*
Blank	<LOQ	-	-
300 (initial 50 ml discarded)	75.31	75.31	85
300 (initial 100 ml discarded)	86.08	86.08	97
300 (centrifugation at 10000 *g*)	84.88	84.88	96
300 (centrifugation at 40000 *g*)	61.90	61.90	70

The amount of test material in solution was shown to be 97 mg/l using the shake flask method followed by filtration (first approximate 100 ml discarded).

Given that all the results obtained were of a similar order and higher than the water solubility value of 60 mg/l supplied by the Sponsor it was considered that this method of preparation was appropriate for use in this study.

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* Calculation based on a 88.82% carbon content.
LOQ = Limit of Quantitation

1,5-Cyclooctadiene (COD): ACUTE TOXICITY TO *DAPHNIA MAGNA*

Appendix 5 Physico-Chemical Measurements

Time Weighted Mean Measured Concentration (mg/l)		0 Hours				24 Hours	48 Hours			
		pH	mg O_2/1	%ASV*	T°C	T°C	pH	mg O_2/1	%ASV*	T°C
Control	R_1	8.0	9.0	99	20.3	20.7	8.0	8.5	96	20.7
	R_2	8.0	9.0	99	20.4	20.7	8.0	8.5	96	20.6
0.0053	R_1	8.0	8.9	98	20.4	20.8	8.0	8.4	94	20.7
	R_2	8.0	8.9	98	20.4	20.8	8.0	8.4	94	20.6
0.0080	R_1	8.0	8.9	98	20.3	20.7	8.0	8.4	94	20.6
	R_2	8.0	8.9	98	20.4	20.6	8.0	8.4	94	20.7
0.010	R_1	8.0	8.9	98	20.3	20.7	8.0	8.4	94	20.6
	R_2	8.0	8.9	98	20.4	20.7	8.0	8.4	94	20.5
0.033	R_1	8.0	8.9	98	20.3	20.8	8.0	8.4	94	20.6
	R_2	8.0	8.9	98	20.4	20.6	7.9	8.5	96	20.6
0.036	R_1	8.0	8.9	98	20.3	20.7	8.0	8.4	94	20.6
	R_2	8.0	8.9	98	20.4	20.6	8.0	8.4	94	20.5
0.065	R_1	8.0	8.9	98	20.3	20.7	8.0	8.4	94	20.6
	R_2	8.0	9.0	99	20.4	20.7	8.0	8.4	94	20.7
0.15	R_1	8.0	8.9	98	20.3	20.8	8.0	8.4	94	20.6
	R_2	8.0	8.9	100	20.5	20.7	8.0	8.5	96	20.7
0.29	R_1	8.0	8.9	98	20.4	20.8	8.0	8.5	96	20.6
	R_2	8.0	8.9	98	20.4	20.7	8.0	8.5	96	20.7
0.58	R_1	8.0	9.0	99	20.3	20.6	8.0	8.6	97	20.6
	R_2	8.0	9.0	99	20.4	20.7	8.0	8.6	97	20.7

*ASV = Dissolved oxygen concentration expressed as a percentage of Air Saturation Value
R_1 - R_2 = Replicates 1 and 2

Appendix 6 Statement of GLP Compliance in Accordance with Directive 2004/9/EC

DH Department of Health

THE DEPARTMENT OF HEALTH OF THE GOVERNMENT OF THE UNITED KINGDOM

GOOD LABORATORY PRACTICE

STATEMENT OF COMPLIANCE
IN ACCORDANCE WITH DIRECTIVE 2004/9/EC

LABORATORY	TEST TYPE
SafePharm Laboratories Ltd. **Shardlow Business Park** **London Road** **Shardlow** **Derby** **DE72 2GD**	**Analytical Chemistry** **Environmental Fate** **Environmental Toxicity** **Mutagenicity** **Phys/Chem Testing** **Toxicology**

DATE OF INSPECTION

30th August 2005

A general inspection for compliance with the Principles of Good Laboratory Practice was carried out at the above laboratory as part of the UK GLP Compliance Programme.

At the time of inspection no deviations were found of sufficient magnitude to affect the validity of non-clinical studies performed at these facilities.

Bryan J Wright 21/11/05.

Mr. Bryan J. Wright
Head, UK GLP Monitoring Authority